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13. ABSTRACT (Maximum 200 Words)
Our aim is to dissect the interactions of p21 (a downstream effector of the tumor suppressor protein p53) with its targets, cyclin-cdks and PCNA. In year 2 we have focused on the cyclin-cdks. We have quantitated the contribution of the cyclin binding Cy motif (on cdk substrates) to their interaction with the cyclin-cdks. We find that a Cy motif improves the Km of a substrate by 500-1000 fold without any effect on the Vmax of the peptide or the Km of ATP. Such a large contribution of the Cy motif to substrate recognition explains why a peptide based on the Cy motif of p21 inhibits phosphorylation by cdk. A small chemical that mimics the structure of the Cy motif would be a specific inhibitor of cdks and suppress breast cancer cell growth. Towards this end, site directed mutagenesis has been used to generate a library of mutants in the Cy motif of p21. Using this library we find that the first arginine (R1) and the third leucine (L3) of the Cy motif (RRLFG) are critical for docking to cyclins. The R cannot be efficiently substituted by other basic amino acids and the L cannot be efficiently substituted by other non-polar or hydrophobic residues. The R and L need to be separated by a distance of at least two peptide bonds. These results should help design chemicals that mimic the structure and cdk inhibitory activity of Cy peptides.

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FOREWORD

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INTRODUCTION:

In the introduction to the annual report of '98 we discussed the importance of p53 function in breast cancer and how p53 exerts part of its tumor suppressor activity through the induction of p21. We reported on the domain structure of p21 which allows different portions of p21 to be studied as potential inhibitors of cyclin dependent kinases (cdk) and or the replication factor PCNA. In particular, we focused on the cyclin-binding motif of p21 (the Cy motif) which comprises the sequence RRLFG, because peptides based on this sequence had been shown by us to inhibit cdk.

Cy motif on inhibitors of cdk: The Cy motif was conserved in well known inhibitors of cdk: p21, p27 and p57. Together with our biochemical data, the crystallographic structure of cyclin A-cdk2 complexed with p27, suggests that the Cy motif- cyclin interaction serves as a docking interaction essential for the complete interaction between cdk inhibitors and cyclin-cdk. In this year we have determined how the cyclin binding site of p21 contributes to inhibition of cyclin-cdk kinase, and the implications of this discovery for designing chemicals that mimic p21 action by binding cyclin or cdk2.

Cy motif on substrates of cdk: Cyclin A/cdk2 and cyclin E/cdk2 both play a major role in the G₁/S transition of the cell cycle by the phosphorylation of various substrates including pRB, E2F, and CDC6. Despite their critical role in this process, little is known about how these substrates are targeted to specific cyclin-cdk complexes.

Since the S/T-P-X-K/R consensus phosphorylation site is broadly applicable to all substrates of all cdk, it would be incapable of conferring the substrate specificity seen within a cellular context. An alternate mechanism by which this specificity could be achieved is through the presence of a docking site on the substrate that recruits the appropriate cyclin-cdk to the protein. The resulting high localized concentration of the cyclin-cdk then facilitates the phosphorylation of potential ser/Thr phosphorylation sites that have been brought into close proximity. In previous work, we and others have identified that cyclin-binding (Cy) motifs have been found in substrates such as E2F and CDC6, activators like cdc25A, and inhibitors of the p21/p27 family, and are absolutely required for the association of cyclin-cdk complexes with these proteins (1-3).

The crystal structure of the cdk inhibitor p27 complexed with cyclin A/cdk2 (4) indicates that the N-terminal Cy motif of p27 is bound to a shallow hydrophobic groove on the surface of the cyclin. Although there is no structural evidence to confirm it, it seems likely that substrates containing a Cy motif would bind in a similar fashion as the inhibitor. The Cy motif of the substrate would bind to the same groove on the cyclin and allow potential phosphorylation sites on the protein to associate with the nearby cdk2 subunit and become phosphorylated.

CDC6, a substrate of cdk2 that is dependent on its Cy motif for phosphorylation by cdk2: One substrate which we propose acts in this fashion is the human replication factor, CDC6. This factor is involved in the formation of a pre-replication complex and is required for the initiation of DNA replication (5). At the onset of S-phase, mammalian CDC6 is phosphorylated by cyclin A/cdk2 which inactivates it by exporting it from the nucleus into the cytoplasm (3, 6). Phosphopeptide analysis has shown that this phosphorylation by cyclin A/cdk2 occurs on Ser-54, Ser-74, and Ser-106 (7). This requires the presence of a nearby Cy motif at residues 94-98 as evidenced by the fact that its deletion abolishes phosphorylation at these sites (Delmolino and Dutta, unpublished results).

Kinetic analysis of the contribution of a Cy motif is done more quantitatively if the Cy motif is on a substrate (like CDC6) than on an inhibitor (like p21): Although the kinetic analysis was originally written to study how the Cy motif contributes to the inhibition of cdk by p21, the availability of a substrate that utilizes a similar Cy motif made the analysis much easier. In this year we have performed Lineweaver-Burke analysis of the phosphorylation of substrates with and without Cy motifs and have made the surprising discovery that the Cy motif make a 500-1000 fold to the K_m of the substrate. Such a large effect strongly suggests that the Cy motif is part of a bipartite (the other being S/TPXX/R) substrate recognition sequence for cyclin dependent kinases. A peptide containing the Cy motif of p21 specifically inhibited substrates that used Cy motifs. Thus at the end of year 2 we now have a quantitative value for the contribution of the Cy motif to the interaction of a molecule (a substrate) with the cyclin-cdk (originally to be covered as Task 2 (months 24-36)) and can use this same kinetic system to analyze the mechanism by which p21 inhibits the kinase (Task 1, months 12-24)

BODY

Statement of Work:

Technical objective 1. Analysis of the interaction between p21, cyclin and cdk.

Task 1: Months 1-24: Oligonucleotide directed mutagenesis of the portion of p21 in the cyclin binding site to determine what sequence feature is essential for binding to cyclins.

Completed

Task 1: Months 1-24: Lineweaver-Burke analysis of the inhibition of cyclin E-cdk2 by intact p21 and by p21 without the cyclin binding site.

Postponed to months 24-36

Task 2: Months 24-36: Determination of the binding affinity of the cyclin-binding site for cyclins and of the cdk2 binding site for cdk2 and comparison with the binding affinity of intact p21 with the cyclin E-cdk2 holoenzyme.

Completed early. Using a substrate with and without a cyclin binding motif we now have a quantitative value of the contribution of a Cy motif for binding to cyclin E-cdk2 and cyclin A-cdk2.

Task 3: Months 24-48: Creation and testing of mutations of p21 with different affinities for cyclins D1 and E.

Task 4: Months 24-48: Creation and testing of versions of p21 with variation of the distance between the cyclin-binding and the cdk2 binding sites (in cis).

Technical objective 2. Analysis of the interaction between p21, PCNA and Fen1.

Task 5: Months 1-12: Determination of which part of PCNA interacts with p21 and with Fen1.

Completed.

Task 6: Months 12-24: Creation of p21 derivatives and determination of their IC90 on the processivity of PCNA-polymerase delta.

Task 7: Months 24-36: Creation and testing of PCNA mutants that have lost the interaction with Fen1 but can still stimulate polymerase delta

Tasks 6 and 7 modified based on new information: Months 24-48: Designing D amino acid based retro-inverse peptides that mimic the PCNA interacting portion of p21. Analysis of their ability to inhibit PCNA in vitro.

Task 8: Months 36-48: Testing effect of adding Fen1 to replication reactions or over-expressing Fen1 in MCF-7 cells in culture.

METHODS.

Expression and purification of cyclin/cdk complexes. Baculoviruses expressing GST-cyclin E, GST-cyclin A, and cdk2 were gifts from Helen Piwnicka-Worms. SF9 cells were co-infected with the appropriate cyclin/cdk pair and affinity-purified as previously described (8) with the following changes. After affinity binding to glutathione agarose beads, the complexes were cleaved from GST using Novagen's Thrombin Cleavage Capture Kit.

Mutagenesis of the Cy motif of p21.

The cassette based strategy described in 1998 produced the library of mutants that we needed. PCR-based mutagenesis of GEX-p21N introduced a HindIII site at the N terminal end of the Cy motif (a silent mutation). Together with a naturally occurring BlnI site on the C terminal side of the Cy motif, this allowed us to excise the wild-type Cy motif. Synthetic oligonucleotides were annealed so that a mutant Cy motif was encoded by the cassette and cloned between the HindIII-BlnI sites of GEX-p21N. All candidates were first screened for the addition (or removal) of a restriction enzyme site and then sequenced to determine exactly what amino acid was substituted in the Cy motif. In the RRLFG motif mutations were made that changed each of the residues in turn to A (alanine). R1 and L3 (of the RXL motif) were systematically changed to a battery of amino acids using cassettes degenerate at the relevant sites. Finally selected mutations were made that change two amino acids in tandem.

Peptide Synthesis and purification. Peptides CDC6(tr), PS100, and DTM101 were purchased from Research Genetics Inc. and purified using reverse phase HPLC. CDC6(wt) and CDC6(mut) peptides were synthesized using the Trp-LE expression system (9). Cassettes based on CDC6 were constructed using appropriate primers of CDC6 by polymerase chain reaction and subcloned into the vector pMM (a gift from Stephen Blacklow). The resulting protein is expressed as a fusion protein with a trpLE peptide leader sequence enabling purification by inclusion bodies (9). Peptide purity was assessed by HPLC and identity confirmed by MALDI-TOF mass spectroscopy. The sequences of PS100 and DTM101 are ACRRLFGPVDSE and ACRFGRLPVDSE, respectively. The sequences of the CDC6-derived peptides are shown in Fig. 1.

Kinase assays. Phosphorylation reactions were performed in a total volume of 15 μ l containing 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.02% Triton X-100, 1 μ Ci [γ - ^{32}P] ATP, and various concentrations of ATP and peptide dissolved in deionized water. Five different peptide and ATP concentrations were used that ranged from 0.5 x Km to 5 x Km for each substrate. Reactions were initiated by addition of 1 μ l of enzyme diluted in reaction buffer and incubated at 30°C for 20 min. Reactions were terminated with 1 μ l of 0.5 M EDTA and 10 μ l of the reaction mixture spotted onto a 2 cm x 2 cm square of Whatman P81 phosphocellulose filter paper. Papers were washed in 0.5% H_3PO_4 three times for five minutes, once in 50% EtOH, 0.5% H_3PO_4 for five minutes, and dried under a heat lamp.

Incorporation of [γ - ^{32}P] ATP into the phosphoacceptor peptide was then quantified by liquid scintillation counting of the paper squares. Under these conditions less than 10% of peptide was phosphorylated upon termination of reaction and velocities were linear with respect to both time and enzyme concentration.

Kinetic analysis. Assuming steady state kinetics, initial velocity data and ATP concentrations were fitted to the Michaelis-Menten equation [1] using the Kaleidagraph program.

$$v_0 = \frac{V_{\max}^{\text{app}} [\text{ATP}]}{K_m^{\text{app}} + [\text{ATP}]} \quad [1]$$

where V_{\max}^{app} and K_m^{app} represent the apparent kinetic parameters at a single peptide concentration. A plot of $1/v_0$ versus $1/[\text{ATP}]$ yields a line with slope of $K_m^{\text{app}} / V_{\max}^{\text{app}}$ and intercept $1/V_{\max}^{\text{app}}$. Data collected at several peptide concentrations were plotted on double reciprocal plots ($1/v_0$ versus $1/[\text{ATP}]$) and modeled according to the equation [2].

$$\frac{1}{v_0} = \frac{1}{V_{\max}} + \frac{K_m^{\text{ATP}}}{V_{\max} [\text{ATP}]} + \frac{K_m^{\text{peptide}}}{V_{\max} [\text{peptide}]} + \frac{K_x}{V_{\max} [\text{ATP}] [\text{peptide}]} \quad [2]$$

Based on equation [2], the slope and intercept of each line can be represented by equations [3] and [4], respectively.

$$\text{slope} = \frac{K_m^{\text{ATP}}}{V_{\max}} + \frac{K_x}{V_{\max}} \left(\frac{1}{[\text{peptide}]} \right) \quad [3]$$

$$\text{intercept} = \frac{1}{V_{\max}} + \frac{K_m^{\text{peptide}}}{V_{\max}} \left(\frac{1}{[\text{peptide}]} \right) \quad [4]$$

Final V_{\max} and K_m values were determined using plots of slope and intercept versus inverse peptide concentration. V_{\max} was divided by enzyme concentration to calculate K_{cat} . All experiments were done at least twice in duplicate. Protein quantitation was determined by Bio-Rad protein assay.

RESULTS AND DISCUSSION

Purification of Enzymes and Substrates – To determine the contribution of the Cy motif to a cyclin/cdk substrate, we constructed a series of recombinant peptide substrates derived from the replication factor, HsCDC6 (3, 10). These peptides all contain a cyclin/cdk phosphorylation site at the N terminus and either a wild type Cy motif (CDC6(wt)), a mutated Cy motif (CDC6(mut)) or a truncation at the C-terminus (CDC6(tr)). We postulated that these peptides would be ideal substrates for this study considering that the N terminal SPXK is known to be phosphorylated in vitro and (2) the phosphorylation of this site in vivo is dependent upon an intact Cy motif ((7), Delmolino and Dutta, unpublished results). The two sites are in close proximity in the amino acid sequence of HsCDC6 (~ 30 residues) allowing a peptide to easily span this region. After expression of these peptides in *E. coli*, they were purified to homogeneity before their use in the kinetic studies (data not shown). Cyclin A/cdk2 and cyclin E/cdk2 were also purified to homogeneity as determined by SDS-PAGE and Coomassie Blue staining (Fig 2). The identities of the proteins were confirmed by western blotting with the appropriate antibodies (data not shown)

Determination of Kinetic Parameters. Using purified enzyme and the peptide substrates, we developed a highly reproducible kinase assay. Phosphorylation of the peptide substrates by both cyclin A/cdk2 and cyclin E/cdk2 followed hyperbolic kinetics

and increased linearly as a function of both enzyme concentration and time when substrate concentrations were not limiting (data not shown). All further experiments were carried out using conditions within this linear range to ensure the results could be interpreted using Michaelis – Menten based equations.

Initial velocities were determined for both cyclin A/cdk2 and cyclin E/cdk2 complexes using our CDC6-based peptides as substrates. These velocities were plotted against ATP concentrations on a double-reciprocal plot using various fixed concentrations of peptide substrate. A representative plot in which cyclin E/cdk2 was used to phosphorylate the CDC6(wt) peptide is shown in (Fig 3A). In all of these plots, the intersecting pattern of initial velocities is consistent with a sequential kinetic mechanism in which both substrates (ATP and peptide) must be bound before any products are released. From this data, however, we are unable to show whether substrate addition is an ordered or random process. K_{cat} and K_m for a given substrate/enzyme pair were determined by secondary re-plots of the slopes and intercepts of the initial velocity lines versus substrate concentration (Fig 3, B and C). A summary of the data for all of the enzymes and substrates can be found in Table 1.

The wild-type substrate was efficiently bound by both cyclin A/cdk2 and cyclin E/cdk2 as demonstrated by K_m values of 1.7 μ M and 7.9 μ M, respectively. Upon deletion of the Cy-motif containing C-terminus, these values increased 500-fold to 1.4 mM for cyclin A/cdk2 and 2000-fold to 17 mM for cyclin E/cdk2. These dramatic increases in K_m demonstrate the importance of the Cy motif in targeting substrates to these enzyme complexes. The K_m values for CDC6(mut) were 27 μ M and 165 μ M for cyclin A/cdk2 and cyclin E/cdk2, respectively, a 15-fold and 20-fold increase compared to the wild-type peptide. Thus, this mutation produces a partially functional Cy motif, rather than a completely non-functional motif.

In contrast to the K_m values for the peptide substrates, The K_{cat} and the K_m^{ATP} values for the enzymes remained very similar with less than a 2-fold change between substrates. This would suggest that although the Cy motif plays a critical role in increasing the affinity of cyclin/cdk complexes for a particular substrate, it does not increase the efficiency of phosphoryl transfer from ATP to the peptide.

Competition with Cy-motif containing Peptides. To further demonstrate that the Cy motif acts as a docking site for the interaction of substrate with enzyme, we tested the ability of a Cy-motif containing peptide, PS100, to inhibit the phosphorylation of our peptide substrates. If the Cy motif truly directs substrates in this manner, then the PS100 peptide is expected to inhibit the phosphorylation of Cy motif-containing substrates such as our CDC6(wt) and CDC6(mut) peptides but unable to inhibit CDC6(tr) which lacks a Cy motif. The data are shown in Figures 4A and 4B. The concentration of the substrate peptides had to be adjusted to obtain equivalent phosphorylation by cyclin/cdk complexes, with more of CDC6(tr) being used relative to CDC6(wt) or CDC6(mut). Despite this, a comparison of the ratio of the inhibitor to substrate for any given peptide substrate shows that PS100 selectively inhibits the phosphorylation of only Cy motif containing substrates, CDC6(wt) and CDC6(mut), but not that of CDC6(tr). The data also show that PS100 is a far more effective inhibitor of cyclin E/cdk2 than cyclin A/cdk2 (~50 fold lower IC_{50}) when using CDC6(wt) as the substrate. Interestingly, when the PS100 peptide is used in combination with CDC6(tr), there appears to be a 60% increase in kinase activity. The source of this activation is unclear. DTM101, a peptide containing a scrambled Cy motif, does not inhibit the phosphorylation of any of the substrates (data not shown), consistent with our previous results that a negative control inhibitory peptide containing a mutation in the Cy motif does not inhibit the phosphorylation of Rb (1).

Mutagenesis of the Cy motif of p21. Table 2 summarizes the IC₅₀s (concentration of p21 at which kinase activity is decreased to 50%) of all the p21N derivatives on cyclin E-cdk2 and cyclin A-cdk2. For reference, deletion of the Cy motif of p21N causes the IC₅₀ on cyclin E-cdk2 to increase to 1000 nM, and that on cyclin A-cdk2 to increase to 60 nM. The following patterns emerge upon examination of the mutations in RRLFG:

Systematic change of each residue to alanine suggests that R1 and L3 are most important because changing either of them had the most profound effect on the IC₅₀. This might explain the conservation of RXL in most Cy motifs identified to date.

R2 is important for cyclin E but not for cyclin A. The same holds true for G5. Thus Cy motifs can be designed that have decreased affinity for cyclin E but not cyclin A.

Although R1 is important, certain substitutions in this position are well tolerated: R1V and R1F interact almost like wild type p21N with cyclin A-cdk2. Given the hydrophobic nature of the docking site on the cyclin A (whose crystal structure has been solved), this result is interesting. The inability of cyclin E-cdk2 to tolerate the same mutations suggest that the docking site on cyclin E is significantly different.

Although R1 is important, a basic charge in this position is not sufficient given the poor activity of R1K and R1N on cyclin A-cdk2 (almost the same effect as a deletion of the Cy motif). R1K and R1N are partially active on cyclin E-cdk2 (70-100 nM, while the deletion of the Cy motif increases the IC₅₀ to 1000 nM) suggesting that one could design Cy motifs that are partially active on cyclin E-cdk2 but not on cyclin A-cdk2.

Although L3 is important, other hydrophobic or nonpolar residues in this position do not allow an interaction with cyclin A. Interaction with cyclin E can partially tolerate a valine in this position(L3V)

Exploiting the p21-PCNA-Fen1 interactions to develop chemicals that will inhibit breast cancer cell growth.

Tasks 6 and 7 of technical objective 2 were directed at developing the p21-PCNA interaction into a possible therapeutic chemical that would selectively permit PCNA-Fen1 interaction to persist while the PCNA-polymerase interaction is interrupted. We had hypothesized that introduction of such an engineered p21 into breast cancer cells will promote unbalanced exonuclease activity of Fen1 (without the compensatory polymerase activity) resulting in excess DNA damage to the cell and cell death.

Last year we reported that the QLGI motif in the inter-domain connector loop of PCNA interacts with p21. Several groups have characterized exactly which portions of PCNA interact with polymerase delta and with Fen1 (11-14). The reports provide strong evidence that the same QLGI motif of PCNA is involved in these interactions. Thus it is unlikely that we will succeed in our original goal of obtaining selective mutants in p21 which will spare the PCNA-Fen1 interaction while disrupting the PCNA-polymerase delta interaction. Whenever p21 associates with PCNA, both Fen1 and polymerase delta will be displaced from the PCNA.

In light of this we decided to combine Tasks 6 and 7 (months 12-36) to develop the peptide based on the C terminus of p21 as a possible therapeutic agent for breast cancers. Since we now know that many cellular proteins interact with the QLGI motif of PCNA (p21, polymerase delta, Fen1, DNA ligase I, DNA methyl transferase, chromatin assembly factor), any stable peptide that interacts with this portion of PCNA will have profound growth inhibition in proliferating cells. Below is an alignment of the PCNA interacting domains of several of these proteins:

p21 human	RR	Q	TS	M	TD	FY	HS
p21 mouse	RR	Q	TS	L	TD	FY	HS
Fen1 human	ST	Q	GR	L	DD	FF	KV
Rad2 pombe	IP	Q	GR	L	DS	FF	KP
Rad27 cerevisiae	GI	Q	GR	L	DG	FF	RL
XP-G human	QT	Q	LR	I	DS	FF	RL
MCMT human	TR	Q	TT	I	TS	HF	AK
MCMT mouse	TR	Q	TT	I	TA	HF	TK
MCMT chicken	GR	Q	PT	I	LS	VF	SK
LigI human	M	Q	RS	I	MS	FF	HP
LigI mouse	M	Q	RS	I	MS	FF	QP
LigI frog	M	Q	RT	I	KS	FF	QP

This PCNA-interacting consensus peptide sequence (QXXMXXFY) is contained in a 39 amino acid p21C2 peptide and a 20 amino acid peptide-70 (SQGRKRRQTSMDFYHSKRR) used by us and another group to inhibit PCNA. Although both peptides of p21 interacted with PCNA and efficiently inhibited its activity in vitro, the p21C2 peptide (which contains the entire sequence of peptide-70) was only weakly effective at suppressing cell growth (15, 16). We concluded that instability of the peptide in vivo was responsible for this weak effect. A new approach suggests that we can now improve on the PCNA interacting peptide (QXXMXXFY) so that it will be stable inside cancer cells and inhibit PCNA activity. The modified tasks 6 and 7 will address these experiments. The end goal remains the same: to utilize the p21-PCNA-Fen1 interaction to develop a small chemical that will inhibit breast cancer cell growth.

Modified task 6 and 7 (months 24-48): In these modified tasks we wish to explore whether chemically synthesized PCNA binding peptides made with D amino acids have an activity similar to the traditional L amino acids. Since biological systems have evolved almost exclusively with L amino acids, most biological proteases are unable to degrade the D amino acid peptides. D peptides are occasionally less immunogenic than L peptides and their retention time in the body prolonged relative to L peptides making them suitable for development as anti-proliferative agents. Of course, the inverted chirality of the D amino acids means that the positions of the side chains are altered relative to the L amino acids. For the amino acid side chains on a D peptide to present the same active sites in approximately the same positions as the L peptide sequence, one has to synthesize the D-peptide in the retro direction: the N-C order of amino acids is reversed.

This peptidomimetic approach has worked in several circumstances (17-22). For example certain antibiotics synthesized with D amino acids exhibit similar potency of activity as those synthesized with L amino acids. Monoclonal antibodies raised against a retro-inverse peptide recognize the L amino acid peptide with reasonable affinity. The retro-inverse approach is most likely to work when the peptide is relatively unstructured, and in this respect the PCNA interacting QXXMXXFY motif is ideal because of its random coil structure (15).

We will purchase two retroinverse peptides based on the PCNA interacting portion of p21:

dRdRdKdSdHdYdFdDdTdMdSdTdQdRdRdKdRdGdQdS
dSdHdYdFdDdTdMdSdTdQdRdR.

We will use exactly the same methods described in the project application to determine whether these D peptide inhibits the interaction of PCNA with Fen1 and with polymerase delta in vitro.

Since the essential assays were described in the original grant application (we have only modified the reagents to be used based on newly published information) we can complete our tasks and reach our objective without any change of budget.

CONCLUSIONS

The Cy motif is part of a bipartite substrate recognition sequence for cdks. We have used a series of peptide substrates derived from HsCDC6 to determine the contribution of a Cy motif to the phosphorylation of a substrate by cyclin/cdk complexes. This detailed kinetic analysis of the phosphorylation of these substrates reveals its importance in substrate recognition by cyclin/cdks and provides additional insight into its mechanism of action.

The CDC6 wild type peptide was efficiently phosphorylated *in vitro* by both cyclin E/cdk2 and cyclin A/cdk2 complexes. The measured K_m for the peptide was less than 10 μM for both enzymes suggesting the existence of a high affinity interaction between the enzyme and our substrate. This is in contrast to previously characterized substrates whose K_m values were no lower than 200 μM – 100-fold greater than our peptide (23). Since these previously characterized substrates contained only the consensus S/T-P-X-K/R phosphorylation site, this reduction in K_m for our peptides can likely be attributed to the presence of a Cy motif. Indeed, the presence of this Cy motif makes the wild-type CDC6 peptide the most efficient peptide substrate of cyclin/cdk complexes characterized to date. The extremely efficient phosphorylation of our CDC6(wt) peptide is surprising considering a study by Solomon *et al.* which defined the sequence requirements of the consensus cdk phosphorylation site (23). They showed that a SPPK phosphorylation site, like that present in CDC6, is phosphorylated at less than 5% of the level of the SPRK phosphorylation site of their wild-type peptide. This decrease in phosphorylation can be attributed to the enzyme's inability to tolerate a proline at the third position of the sequence. Their result is consistent with our data in which the K_m of the truncated peptide containing the SPPK site was in the millimolar range for all cyclin/cdk complexes tested. Hence, we conclude that the addition of a Cy motif is sufficient to convert a peptide whose phosphorylation site would normally make it a poor substrate into a very efficient substrate, emphasizing the contribution of the Cy motif to the enzyme-substrate interaction. Therefore, substrate recognition by cyclin/cdks occurs through a bipartite recognition sequence on the substrate consisting of both the cdk phosphorylation site (S/T-P-X-K/R) and the cyclin binding Cy motif.

Our results very clearly show that the mechanism for increased phosphorylation of the Cy motif-containing peptide occurs through a large decrease in K_m for the peptide substrate. V_{max} and K_m^{ATP} , however, remained relatively unchanged in our studies. This is consistent with the Cy motif acting as a docking site that targets the substrate to the enzyme primarily by increasing the affinity of the interaction between the two proteins. In an earlier study, Harlow and co-workers demonstrated that the Cy-cyclin interaction could be substituted by engineering a separate docking interaction between the cyclin and the substrate (24). Our results kinetically confirm their conclusion that the Cy-cyclin interactions serves to increase phosphorylation by bringing the substrate in close proximity to the enzyme.

A peptide containing the Cy motif of p21 specifically inhibits substrates with Cy motifs: We had earlier reported that the Cy motif of p21 inhibited the phosphorylation of pRB but not histone H1 (1). Now we show that a Cy motif containing peptide (PS100) is able to selectively inhibit only Cy motif containing substrates. This is consistent with PS100 competing with substrate for the binding site on the cyclin and confirms our model in which the Cy motif targets substrates to the enzyme via a docking site on the cyclin. If the physiological targets of cyclin-cdks necessarily use the Cy-cyclin interaction, peptides or chemicals that mimic the Cy motif are likely to be specific inhibitors of cdks and will differ from existing inhibitors that target the ATP binding site. Indeed,

preliminary studies show that such peptides lead to the selective killing of only transformed cells in which the E2F pathway has been deregulated (25).

Curiously, there seems to be a small but reproducible increase (~60%) in the phosphorylation of the truncated CDC6 peptide by cyclin A/cdk2 when PS100 is introduced into the reaction. This opens up the possibility that the binding of a Cy motif to the cyclin leads to a small increase in the catalytic efficiency of the enzyme. This effect, however, is minor compared to the dramatic decrease in K_m caused by the presence of a Cy motif and therefore seems unlikely to play a significant role in the mechanism of action of a Cy motif on this particular enzyme-substrate combination.

Specificity of cyclins for substrates may be determined by the Cy motif.

Not much is known about how the specificity of cyclin/cdk complexes is determined. Our results suggest one mechanism by which this specificity could be achieved. The K_m for CDC6(wt) was 1.7 μM for cyclin A/cdk2 and 7.9 μM for cyclin E/cdk2 suggesting that both enzymes have a high affinity for the Cy motif present in this particular peptide. In contrast, CDC6(mut) had a K_m of 27 μM for cyclin A/cdk2 but 163 μM for cyclin E/cdk2. Therefore, cyclin A/cdk2 but not cyclin E/cdk2 could effectively phosphorylate the mutant substrate. Thus, although the wild-type Cy motif interacted strongly with both enzymes, mutations could be made in the Cy motif which confer specificity to cyclin A/cdk2 over cyclin E/cdk2. We also observed that the inhibitory PS100 peptide containing the RRLFG Cy motif was a far better inhibitor of cyclin E/cdk2 ($IC_{50} \approx 10 \mu M$) than cyclin A/cdk2 ($IC_{50} \approx 500 \mu M$). Based on these results, it seems likely that different Cy motifs will preferentially associate with a specific cyclin/cdk complex and thereby target that substrate for phosphorylation by only that enzyme.

Mutagenesis of Cy motif on p21N: Based on the results in Table 2 we conclude that it should be possible to design chemicals that look like the Cy motif and inhibit cyclin-cdk. The ideal chemical should have a residue like arginine that has an aliphatic chain of three carbons to provide a long enough hydrophobic arm that can interact with the hydrophobic groove on the docking site. At the end of this hydrophobic arm there must be a strong basic residue presumably to interact with the acidic surface that flanks either side of the hydrophobic groove. Lysine (4 carbon aliphatic arm) or asparagine (2 carbon aliphatic arm) did not substitute for arginine indicating that the 3 carbon arm of arginine is just right. However, the positive charge at the end of the aliphatic arm appears important because non-polar or hydrophobic residues could not substitute for R1.

The failure of ARLFG (R1A) to inhibit either of the cyclin-cdks indicates that the arginine like side-chain has to be separated from the leucine by at least two peptide bonds (C-C-N-C-C-N-C, the bold residues bearing the R1 and L3). The partial loss of activity with R2A on cyclin E-cdk2 (and the functionality of RNLFG in p27) suggests that basic residues in the second position may promote the interaction with the cyclin. In the case of cyclin A it appears that the second position acts strictly as a spacer.

L3 is important. A two carbon aliphatic chain with two methyl groups at the end appears to be the ideal structure. Because L3V is partially tolerated by cyclin E, it appears that the two methyl groups at the end of the aliphatic chain is important. The failure of L3F to act as an inhibitor suggests that bulky hydrophobic groups are not helpful in this position.

The crystal structure of p27 with cyclin A-cdk2 indicates that F4 has extensive interaction with the cyclin. Surprisingly, mutation of F4 to alanine had no effect on the interaction of p21N with cyclin A suggesting that in the aqueous environment the exact contact sites may be slightly different than in the packed crystal structure. We find this difference particularly interesting because it validates our approach of systematic mutagenesis of the Cy motif followed by functional assays.

FIGURE LEGENDS

Fig. 1. Schematic of Substrate Peptides Derived from HsCDC6. Peptides were constructed that spanned the consensus cdk phosphorylation site from residues 74-78 and the Cy motif from residues 94-98. The N-terminal cdk phosphorylation site and the C-terminal Cy motif are highlighted in bold.

Fig. 2. SDS-PAGE of cyclin E/cdk2 and cyclin A/cdk2. Purified Cyclin E/Cdk2 (lane 1) and Cyclin A/Cdk2 (lane 2) were loaded on a 12% gel and the proteins were stained with Coomassie Blue.

Fig. 3. Representative Initial Velocity Patterns and Secondary Re-plots. (A) Initial velocity pattern was determined for cyclin E/cdk2 using ATP as the varied substrate and the following fixed concentrations of CDC6 wild-type peptide: 2.5 μ M (●), 5 μ M (×), 10 μ M (▲), 10 μ M (■), and 40 μ M (◆). (B) Secondary plot of primary slopes vs. reciprocal peptide concentration. (C) Secondary plot of primary intercepts vs. reciprocal peptide concentration.

Fig. 4. Cy-motif containing peptide selectively inhibits the phosphorylation of only Cy-motif containing substrates by (A) cyclin E/cdk2 and (B) cyclin A/cdk2. The Cy Motif Containing peptide PS100 is able to inhibit the phosphorylation of 5 μ M CDC6(wt) (●), 50 μ M CDC6(mut) (○) but not 1 mM of CDC6(tr) substrate (■).

Table 1. Kinetic Parameters for cyclin/cdk complexes and CDC6-derived peptides. Units for K_m^{ATP} and $K_m^{peptide}$ are expressed in μM while units for K_{cat} are in pmol of phosphate incorporated per minute per μg of enzyme (pmol/min/ μg).

		CDC6 (wt)	CDC6 (mut)	CDC6 (tr)
CyclinE/cdk2	K_{cat}	1110 ± 64	770 ± 142	1200 ± 425
	$K_m^{peptide}$	7.9 ± 0.53	163 ± 34	$\sim 17000 \pm 6400$
	K_m^{ATP}	206 ± 16	165 ± 32	$\sim 1300 \pm 560$
CyclinA/cdk2	K_{cat}	10.5 ± 0.80	33 ± 2.1	13.8 ± 3.3
	$K_m^{peptide}$	1.7 ± 0.33	27 ± 2.4	1450 ± 620
	K_m^{ATP}	34 ± 13	84 ± 16	95 ± 27

TABLE 2

The IC₅₀ (nM of inhibitor at which kinase activity of indicated cyclin-cdk is inhibited by 50%) of p21N wild type and its derivatives at the Cy motif. Nomenclature: R1A indicates that the first arginine of the RRLFG motif has been changed to alanine.

<u>Inhibitor</u>	<u>Cyclin E/cdk2</u>	<u>Cyclin A/cdk2</u>
p21N (RRLFG)	10	10
p21N ΔCy	1000	60
R1A	300	40
R2A	180	10
L3A	1000	50
F4A	60	10
G5A	100	10
R1M	600	30
R1S	900	50
R1E	400	200
R1W	500	60
R1N	100	50
R1L	175	40
R1V	125	10
R1F	125	20
R1K	70	50
R1Q	380	70
L3H	1000	50
L3T	800	40
L3S	800	60
L3F	800	30
L3R	>1000	60
L3V	60	40
L3A	1000	50
p21N (RRLIF)	60	40
p21N (RREAG)	7200	180
p21N (LALFG)	320	80

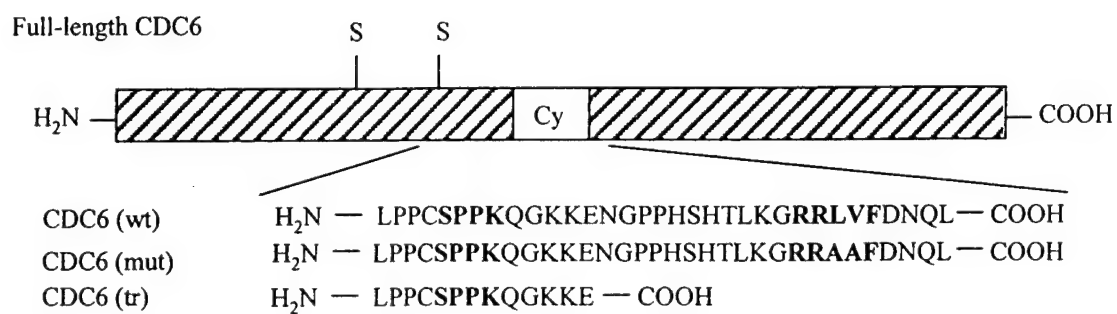


Figure 1

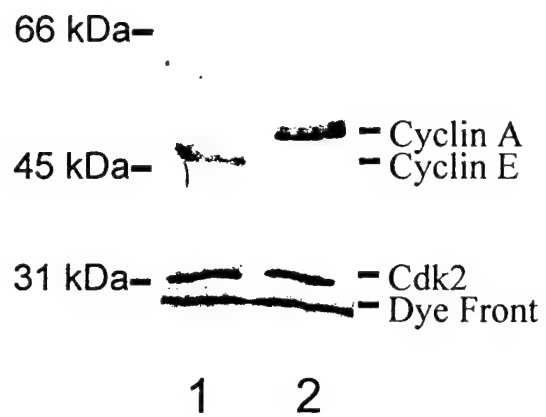


Figure 2

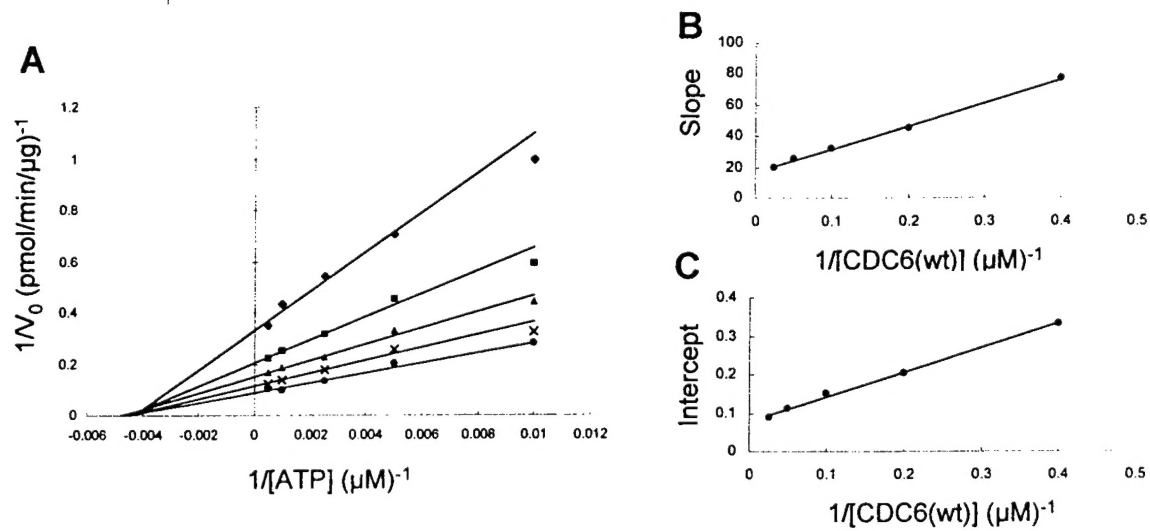


Figure 3

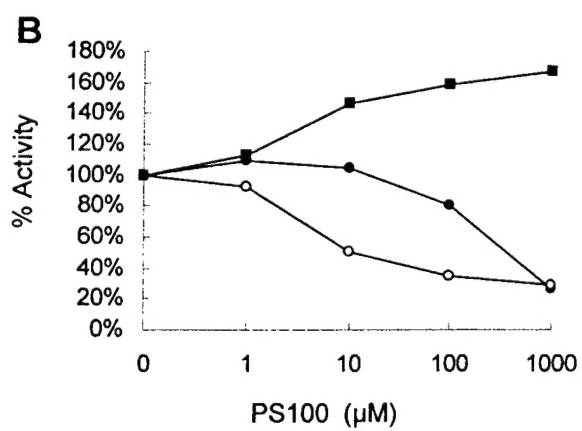
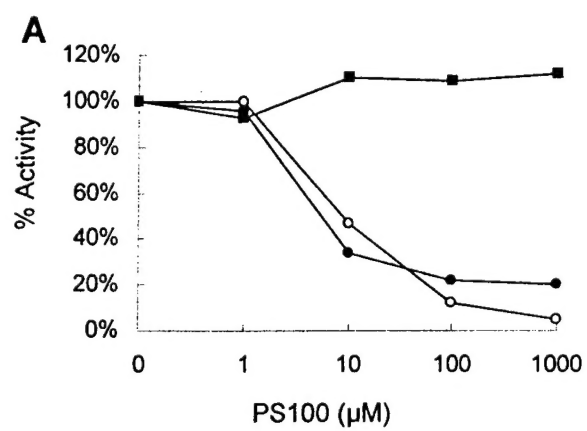


Figure 4

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